The Uptake from the Blood of Triglyceride Fatty Acids of Chylomicra and Low-Density Lipoproteins by the Mammary Gland of the Goat

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Although a portion of the fatty acids of milk triglycerides are known to be synthesized within the mammary gland, part are derived from unknown lipid fractions of the blood (Folley & McNaught, 1961). The experiments reported in the present paper were designed to discover which plasma-lipid fractions provide these fatty acids. It is shown that, in lactating goats, there is an arteriovenous difference across the mammary gland in the triglyceride fatty acids of chylomicra and low-density lipoproteins of density 1·005–1·019 that could account for a large portion of the fatty acids of milk. No significant arteriovenous differences could be detected in the free fatty acids of plasma or in the fatty acids of the low-density lipoproteins of *d* 1·019–1·063 or of high-density lipoproteins. Arteriovenous measurements are also reported for D-β-hydroxybutyrate, acetoacetate and free glycerol.

METHODS

Blood sampling. Arterial and mammary venous blood samples were taken from four Saanen goats at the A.R.C. Institute of Animal Physiology (see Table 1). The animals were being given a diet of hay *ad libitum* and a balanced cereal ration (plus vitamins A and D and minerals) similar to that commonly fed to milking cows and containing about 5% of fat. The cereal ration was given twice daily, when the animals were milked, at about 9·30 a.m. and 5 p.m. The milk yield of each gland was measured separately.

All the goats had been prepared surgically so that blood samples could be obtained from one mammary gland (half the udder) without disturbance in the conscious animal. One or both carotid arteries and one caudal superficial epigastric vein ('milk vein') had been permanently exteriorized as skin-covered loops and the halves of the udder had been separated with skin to divide the blood vessels crossing between the left and right glands, so that venous blood was obtained from one gland only (Linzell, 1960*b*). The exteriorized vein does not always contain purely mammary venous blood owing to the variable degree of incompetence of its valves (Linzell, 1960*a*). To reduce the possibility of obtaining mammary venous blood contaminated with blood from surrounding structures, sampling was done with the animal in the standing position and, with the older animals, the other main veins in the mammary area were manually compressed during the collection of venous blood. From previous work (Linzell, 1960*b*), and from measurements of udder volumes, it may be assumed that at least 90% of the venous blood came from mammary secretory tissue. Sampling was done on the milking stand in the animal house about half an hour after the morning milking. The animals were held by the head collar by their usual milker, but were allowed to continue eating their cereal ration while the samples were taken under local anaesthesia over a period of 5–10 min. Except once, when goat Sally became restless through slow arterial sampling, none of the animals appeared upset.

Table 1. *Goats used for sampling of arterial and mammary venous blood*

The milk yield is the average of 7 days.

	Time and date of sampling	No. of lactation	Week of lactation	Milk yield of gland sampled (l./day)
Jill	a.m. 27. viii. 62	6	25	1·75
Jill	a.m. 24. x. 62	6	33	1·37
Sally	a.m. 27. viii. 62	5	7	2·5
Sally	a.m. 24. x. 62	5	16	1·58
Balham	a.m. 10. xii. 62	1	41	1·08
Hilda	a.m. 10. xii. 62	2	Dry	0

Blood samples, the largest of which (200 ml.) were only 7% of the estimated blood volume, were collected in tubes containing sufficient powdered heparin (Boots Pure Drug Co. Ltd.) to prevent clotting. The blood was immediately centrifuged at 1000 *g* and 0° for 45 min., and the plasma was recovered. Portions were frozen until they could be analysed for β-hydroxybutyrate, acetoacetate and glycerol. The remainder was kept at 0° for not longer than 8 hr. before beginning the separations of chylomicra and lipoproteins, and the assays of clearing-factor lipase and free fatty acids.

Preparation of chylomicron and lipoprotein fractions. These were separated in the ultracentrifuge by flotation at particular salt densities. A known volume of each plasma sample (20–30 ml.) was first adjusted from *d* 1·005 to *d* 1·019 (Bragdon, Havel & Boyle, 1956). It was then spun at 114 000 *g* and 4° for 24 hr. in celluloid tubes of 6·5 ml. capacity in the no. 40·3 head of the Spinco (model L) ultracentrifuge. Chylomicra and low-density lipoproteins of $d < 1{\cdot}019$ were recovered from the top of each tube by slicing it 2 cm. from the base of the liquid meniscus with a Spinco tube slicer. The cap and severed top of the centrifuge tube were washed carefully with water and the washings added to the lipid fraction. Any aggregates of lipid in the fraction were dispersed by forcing it several times from a hypodermic syringe through a no. 28 needle. The liquid remaining in the centrifuge tube contained all lipoproteins of $d > 1{\cdot}019$: that is, the low-density lipoproteins of *d* 1·019–1·063 and the high-density lipoproteins. The sum of the volumes of the two fractions, before addition of the washings, was always slightly less than the original volume owing to leakage during centrifuging and slicing the tube. The loss was always less than 10%. It was assumed to have occurred to each fraction in proportion to its volume, and the final analyses were corrected accordingly.

In two experiments the chylomicra and $d < 1{\cdot}019$ low-density lipoproteins were fractionated further. Known portions of the solutions containing them were adjusted to *d* 1·005 by adding water and then spun at 114 000 *g* and 4° for 18 hr. By slicing the tubes as before, chylomicra at the top were separated from lipoproteins (*d* 1·005–1·019) at the bottom.

Analyses

Samples of the lipid fractions were blown into 50 vol. of ethanol–ether (3:1, v/v). The mixture was heated to boiling, filtered while hot, and the precipitate on the paper washed with half the original volume of ethanol–ether. The total filtrate was evaporated, the residue redissolved in 3 ml. of ethanol–ether and esterified fatty acids were determined by the method of Stern & Shapiro (1953).

Phosphatide was determined as follows. A portion of the lipid fraction was blown into 20 vol. of ethanol–acetone (1:1, v/v). The mixture was heated to boiling, cooled, filtered, and the residue on the filter paper washed with one-quarter of the original volume of ethanol–acetone. The total filtrate was evaporated in micro-Kjeldahl flasks and digested with perchloric acid. The liberated inorganic phosphate was determined by the method of Allen (1940) and the weights were multiplied by 25 to give those of phosphatide.

Total cholesterol (free plus esterified) was determined by the method of Henly (1957) after dialysis of the lipid fractions for 8 hr. against a large volume of distilled water.

Free fatty acids of plasma were determined by the method of Dole (1956) as modified by Salaman & Robinson (1961).

All the above analyses were made in quadruplicate. The mean values are quoted and these differed from the individual values by less than 5%. The fatty acids were assumed to have an equivalent weight of 280.

Individual esterified fatty acids of the chylomicron plus $d < 1{\cdot}019$ low-density lipoprotein fractions were determined by the method of Getz & Bartley (1961).

Clearing-factor lipase of plasma was assayed by measuring the free fatty acids released when the plasma was incubated with chylomicron triglyceride. Plasma (3 ml.) and a rat chyle–serum mixture (1 ml.) were incubated at 37°. Samples (0·5 ml.) were taken in triplicate at 0 and 4 hr. and free fatty acids determined by the method of Salaman & Robinson (1961). The chyle–serum mixture was prepared by adding rat chyle to four times its volume of rat serum, and was left at room temperature for at least 1 hr. The chyle was collected from the thoracic ducts of rats that had been fed with olive oil (French, Robinson & Florey, 1953), and contained 160 μmoles of esterified fatty acid/ml. Serum was obtained by recalcifying citrated rat plasma with M-$CaCl_2$ (0·025 vol.) and removing the clot that formed.

Acetoacetate and D-β-hydroxybutyrate were determined enzymically by the method of Williamson, Mellanby & Krebs (1962).

Glycerol was determined either chemically by the method of Hanahan & Olley (1958) or enzymically with glycerokinase (Wieland, 1962). Both methods gave essentially the same values.

RESULTS

Arteriovenous differences in triglyceride fatty acids of chylomicra and lipoproteins. In Table 2 are shown the concentrations of the esterified fatty

Table 2. *Concentrations, in arterial and mammary venous plasma of goats, of the esterified fatty acids of the chylomicra plus* $d < 1{\cdot}019$ *low-density lipoproteins*

Goat	Date	Esterified fatty acids (mg./100 ml. of plasma) Arterial	Venous	Arteriovenous difference
Sally	24. x. 62	20·2	9·0	11·2
Jill	24. x. 62	28·2	6·8	21·4
Balham	10. xii. 62	17·2	8·1	9·1
Hilda*	10. xii. 62	11·4	11·8	−0·4

* Non-lactating goat.

acids of the chylomicra plus $d < 1{\cdot}019$ low-density lipoproteins in the arterial and mammary venous plasma of three lactating goats and one 'dry' goat. In the lactating goats the venous concentrations were less than half the arterial; in the 'dry' goat there was no significant difference. These arteriovenous differences must be almost entirely in triglyceride fatty acids rather than in the fatty acids of phosphatides or cholesterol esters. Thus the concentrations of phosphatide of the same lipid fraction in the arterial and venous plasma of one lactating goat (Sally) were 4 and 3 mg./100 ml. respectively, and of another (Jill) were 6 and 3 mg./100 ml. (phosphatides contain about 75% of fatty acids). The arterial and venous concentrations of cholesterol of the same lipid fraction in another lactating goat (Balham) were 5·0 and 4·9 mg./100 ml. (cholesterol is combined with about one-third of its weight of fatty acids in cholesterol esters).

Table 3 shows the concentrations of certain esterified fatty acids of the chylomicron plus $d < 1{\cdot}019$ low-density lipoprotein fraction in the arterial and venous plasma from the goat Balham. The arteriovenous difference was largely in palmitic acid and stearic acid. Analyses on samples from three other goats confirmed this.

The combined chylomicron plus $d < 1{\cdot}019$ low-density lipoprotein fraction from two goats was further separated into fractions of $d < 1{\cdot}005$ (chylomicra) and d 1·005–1·019 low-density lipoproteins. There was a marked arteriovenous difference in both these fractions, and triglyceride fatty acids from both are taken up by the gland (Table 4).

Table 5 shows the concentrations of esterified fatty acids of the $d > 1{\cdot}019$ lipoproteins in three pairs of arterial and mammary venous plasma samples from two lactating goats. The concentrations are much higher than those of the fatty acids of chylomicra and $d < 1{\cdot}019$ low-density lipoproteins, and arteriovenous differences which could account for a significant part of the fatty acids of milk are more difficult to detect. However, it does not appear that the lactating gland absorbs fatty acids from this fraction in amounts comparable with those absorbed from the fractions of lower density.

Although results have been given for only three lactating goats, measurements on four other goats, with a different fractionation scheme, support the conclusions that have been drawn. Thus in one experiment a chylomicron fraction, and a total low-density lipoprotein fraction of d 1·005–1·063, were recovered separately. The arterial and venous concentrations of the esterified fatty acids of the chylomicron fraction were 7·1 and 3·1 mg./100 ml. respectively, and of the low-density lipoprotein fraction of d 1·005–1·063 were 36 and 26 mg./100 ml. The concentrations of the esterified fatty acids of the high-density lipoproteins (d 1·063–1·21), which were also recovered, were 80 mg./100 ml. in the arterial plasma and 84 mg./100 ml. in the venous plasma.

Arteriovenous differences in the numbers of chylomicra. The plasma samples were examined under the microscope by dark-ground illumination, when the chylomicra appeared as small, bright particles in brownian movement (Frazer & Stewart, 1937). The number of particles in arterial plasma from lactating goats was much greater than that in mammary venous plasma. It was also possible with the naked eye to distinguish the arterial samples from the venous by their extra cloudiness, which was presumably caused by suspended chylomicra. No differences could be seen between the plasma samples from the 'dry' goat either under the microscope or with the naked eye.

Arteriovenous differences in free fatty acids. Free fatty acid concentrations in the arterial and mam-

Table 3. *Concentrations, in arterial and mammary venous plasma of the goat Balham, of the principal esterified fatty acids of the chylomicra plus $d < 1{\cdot}019$ low-density lipoproteins*

	Concn. in plasma (mg./100 ml.)		
Fatty acid	Arterial	Venous	Arteriovenous difference
Lauric	< 0·26	< 0·26	< 0·26
Myristic	< 0·26	< 0·26	< 0·26
Palmitic	4·9	1·9	3·0
Stearic	4·8	1·7	3·1
Oleic	2·7	1·8	0·9
Linoleic	2·0	1·4	0·6

Table 4. *Concentrations, in arterial and mammary venous plasma of lactating goats, of the esterified fatty acids of the chylomicron and of the d 1·005–1·019 low-density lipoprotein fractions*

		Esterified fatty acids (mg./100 ml. of plasma)					
		Chylomicra			Lipoproteins		
Goat	Date	Arterial	Venous	Arteriovenous difference	Arterial	Venous	Arteriovenous difference
Jill	24. x. 62	8·1	3·4	4·7	14·6	2·0	12·6
Balham	10. xii. 62	5·5	1·7	3·8	8·4	5·0	3·4

mary venous plasma of three lactating goats are shown in Table 6. It may be concluded that there were no arteriovenous differences of magnitude comparable with those in triglyceride fatty acids.

Arteriovenous differences in D-*β-hydroxybutyrate, acetoacetate and glycerol.* These arteriovenous differences are shown in Table 7 for plasma samples from three lactating goats. There is a marked and consistent arteriovenous difference in β-hydroxybutyrate, the venous concentration being always less than 40 % of the arterial. This confirms, with a more specific analytical method, the arteriovenous difference in β-hydroxybutyrate that was discovered by Shaw & Knodt (1941) across the mammary glands of lactating cows. No arteriovenous differences of any magnitude are apparent in acetoacetate or free glycerol.

Arteriovenous differences in clearing-factor lipase activity. Clearing-factor lipase has been found in the mammary gland of guinea pigs and rats during lactation (McBride & Korn, 1963; Robinson, 1963*a*), and it may be concerned in the uptake of the triglyceride fatty acids of chylomicra and low-density lipoproteins demonstrated above. It has been suggested that small amounts of the enzyme are liberated into the blood from tissues rich in it (Robinson, 1960). In this study the lipase activities of mammary venous plasma samples from two lactating goats, expressed as μmole of free fatty acid liberated from an added triglyceride substrate/ml. of plasma/hr., were 0·22 and 0·38, and those of the respective arterial plasma samples were 0·07 and 0·08. In the presence of 0·8 M-sodium chloride the release of free fatty acids was completely inhibited, indicating that the lipase was clearing-factor lipase (Korn, 1955).

Table 5. *Concentrations, in arterial and mammary venous plasma of goats, of the esterified fatty acids of the lipoproteins of* $d > 1{\cdot}019$

Goat	Date	Esterified fatty acids (mg./100 ml. of plasma) Arterial	Venous
Sally	24. x. 62	146	148
Jill	24. x. 62	143	148
Balham	10. xii. 62	159	161

Table 6. *Concentrations of free fatty acids in arterial and mammary venous plasma of lactating goats*

Goat	Date	Free fatty acids (mg./100 ml. of plasma) Arterial	Venous
Sally	27. viii. 62	17·6	16·0
	24. x. 62	18·3	15·6
Jill	27. viii. 62	10·9	12·6
	24. x. 62	11·0	10·0
Balham	10. xii. 62	20·0	20·8

DISCUSSION

An arteriovenous difference across the mammary gland in a component of blood is difficult to demonstrate convincingly unless it is greater than 20 % of the arterial concentration (Barry, 1961). Our experiments were undertaken because rough calculations suggested that, if a significant portion of the fatty acids of milk came from the chylomicron, low-density lipoprotein or free fatty acid fractions of blood, the arteriovenous differences would be large proportions of the arterial concentrations of fatty acids of these fractions. The results show that there is, in fact, a large fall across the gland in the concentration of triglyceride fatty acids of the chylomicron plus $d < 1{\cdot}019$ low-density lipoprotein fraction.

It is possible to calculate roughly the proportion of fatty acids of milk that could be accounted for by observed arteriovenous differences. Thus Linzell (1960*b*) has shown that, in the goat at full lactation, about 375 vol. of plasma flows through the mammary gland for each volume of milk secreted. Since the arteriovenous differences in Table 2 range from 9 to 21 mg. of esterified fatty acid/100 ml. of plasma it appears therefore that between 3·4 and 8 g. of triglyceride fatty acids is taken up from the blood during the formation of

Table 7. *Concentrations in arterial and mammary venous plasma of lactating goats of* D-*β-hydroxybutyrate, acetoacetate and glycerol*

A, Arterial plasma values; V, venous plasma values; A–V, arteriovenous difference.

		Concn. in plasma (mg./100 ml.)								
		D-β-Hydroxybutyrate			Acetoacetate			Glycerol		
Goat	Date	A	V	A–V	A	V	A–V	A	V	A–V
Sally	27. viii. 62	6·3	2·3	4·0	0·15	0·19	−0·04	—	—	—
	24. x. 62	5·3	1·7	3·6	0·35	0·20	0·15	0·65	0·48	0·17
Jill	27. viii. 62	6·3	2·4	3·9	0·16	0·18	−0·02	—	—	—
	24. x. 62	5·3	1·6	3·7	0·34	0·24	0·10	0·32	0·30	0·02
Balham	10. xii. 62	5·7	2·3	3·4	—	—	—	—	—	—

100 ml. of milk. These quantities tend to be large in relation to the normal milk-fat concentration of $4{\cdot}2 \pm 0{\cdot}74$ g./100 g. in the Institute herd. Possibly, uptake may not be constant throughout the day or part of the fatty acids may be oxidized to provide energy. In any case it seems clear that, in normal goats, the triglyceride fatty acids of chylomicra and low-density lipoproteins must be an important, and possibly the sole, source of those fatty acids of milk that are derived from the lipids of blood.

The arteriovenous differences in individual triglyceride fatty acids of the chylomicron plus $d < 1{\cdot}019$ low-density lipoprotein fraction (Table 3) show that palmitic acid and stearic acid are taken up in the greatest amounts. Since goat's-milk fat contains almost four times as much oleic acid as stearic acid (Hilditch, 1956), these results support other evidence (Lauryssens, Verbeke & Peeters, 1961) for the conversion of stearic acid into oleic acid within the mammary gland.

Our studies provide no evidence on the mechanism of uptake of these triglyceride fatty acids by the gland, although the higher activity of the clearing-factor lipase in the venous than in the arterial plasma and the marked rise in the content of this enzyme in the mammary gland of the guinea pig and rat at parturition (McBride & Korn, 1963; Robinson, 1963*a*) suggest that it may be involved. The small concentration of free glycerol in plasma did not increase as blood passed through the gland (Table 7). This suggests that the glycerol of the triglycerides is taken up by the gland and could contribute to the triglycerides of milk, even though hydrolysis by clearing-factor lipase may occur during uptake.

No arteriovenous difference in free fatty acids was found of a magnitude comparable with that in triglyceride fatty acids. This does not completely exclude the free fatty acids of plasma as a significant source of the fatty acids of milk fat, since it is conceivable that free fatty acids derived from the plasma triglycerides by action of clearing-factor lipase might enter the venous blood. (The possibility that significant amounts of free fatty acids derived from adipose tissue were secreted into the venous blood can be excluded because of the small proportion of this tissue in the gland.) However, the negligible arteriovenous differences are fully consistent with the experiments of Glascock and colleagues (see Glascock, 1958) with labelled fatty acids that suggest that the free fatty acids of plasma do not normally provide a significant portion of the fatty acids of milk fat in the cow. The possibility is not excluded that in an abnormal nutritional state, such as fasting, the gland would take up free fatty acids from the blood (Robinson, 1963*b*).

The mean arteriovenous difference in D-β-hydroxybutyrate of 3·7 mg./100 ml. of plasma is roughly the same as that found by Shaw & Knodt (1941) in lactating cows. It suggests that in the goat about $3{\cdot}7 \text{ mg.} \times 375 = 1{\cdot}4$ g. is available to the gland during the formation of 100 ml. of milk and that β-hydroxybutyrate is therefore an important metabolite. No consistent arteriovenous difference in acetoacetate was found and its concentration in blood is so low that even if it were completely taken up it could not be a quantitatively important metabolite. Also, the arteriovenous differences in free glycerol were far below the amount that would be found if the free glycerol of the blood was an important precursor of the glycerol of milk fat.

SUMMARY

1. Arteriovenous measurements have been made across the mammary glands of lactating goats to discover which fractions of the plasma lipids provide fatty acids for the formation of milk fat. Pronounced arteriovenous differences were found in the triglyceride fatty acids of chylomicra and $d < 1{\cdot}019$ low-density lipoproteins. The fatty acids taken up from this fraction were principally palmitic acid and stearic acid. No significant arteriovenous difference was found in a non-lactating goat.

2. Arteriovenous measurements of free fatty acids showed that they were not taken up by the gland in amounts comparable with those of the triglyceride fatty acids.

3. It is concluded that in the goat a large part, and possibly all, of the fatty acids of milk that come from the blood lipids are derived from the triglycerides of the chylomicra and $d < 1{\cdot}019$ low-density lipoproteins.

4. The activity of clearing-factor lipase in the mammary venous blood of lactating goats was over three times that in the arterial.

5. In lactating goats pronounced arteriovenous differences were found in D-β-hydroxybutyrate, but not in acetoacetate.

6. Arteriovenous measurements of the free glycerol of blood showed that this fraction could not provide a significant part of the glycerol of milk fat.

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The Phospholipids of the Housefly, *Musca domestica*

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Relatively few investigations have been reported on the phospholipid content of insects. A study of the lipids of bee brain was made by Patterson, Dumm & Richards (1945). More recently Wren & Mitchell (1959) have described the fractionation of the lipids of *Drosophila melanogaster*, Bieber, Hodgson, Cheldelin, Brookes & Newburgh (1961) the phospholipids of the blowfly, *Phormia regina*, and Fast & Brown (1962) the lipids of *Aedes aegypti* larvae. The incorporation of [^{32}P]orthophosphate into the phospholipids of *Arctia caia* moths has been studied by Chojnacki & Korzybski (1962). The present paper describes a study of the phospholipids of the housefly, *Musca domestica*, during which evidence was obtained for the presence of a novel phospholipid of the sphingomyelin type. It was possible to study the minor phospholipid constituents by labelling the insect phospholipids with ^{32}P by techniques similar to those described by Winteringham, Bridges & Hellyer (1955) for the labelling of water-soluble phosphorus compounds. Because of the slow rate of turnover of some of the phospholipids it was not possible to use the distribution of ^{32}P radioactivity as a measure of the distribution of phosphorus, so that this was determined by chemical methods. A preliminary account of the phospholipids of the housefly which are stable to both mild alkaline and mild acidic hydrolysis has been given by Crone & Bridges (1962), and the identification of the housefly phospholipids has been briefly reported by Bridges, Crone & Beard (1962).

MATERIALS

Radioactive chemicals. L-[β-^{14}C]Serine (specific radioactivity 30 μc/mg.) and carrier-free [^{32}P]orthophosphate solution in dilute hydrochloric acid (radioactivity 5 mc/ml.) were obtained from The Radiochemical Centre, Amersham, Bucks.

Other chemicals. Silicic acid (100–200 mesh, chromatographic grade) was purchased from L. Light and Co. Ltd., Colnbrook, Bucks. Before use this was heated for 24 hr. at 120°. Sphingosine was prepared from sphingosine sulphate (L. Light and Co. Ltd.) by the method of Brady & Burton (1956). Glycerylphosphorylcholine was prepared from synthetic DL-α-lecithin (L. Light and Co. Ltd.) by alkaline hydrolysis (Dawson, 1960), and a mixture of glycerylphosphorylserine and glycerylphosphorylethanolamine was obtained by a similar hydrolysis of kephalin from natural sources (L. Light and Co. Ltd.). Ethanolamine phosphate and *myo*inositol 2-phosphate were obtained from the California Corp. for Biochemical Research, Los Angeles, U.S.A. *myo*Inositol (under the name 'meso-inositol') was purchased from British Drug Houses Ltd., Poole, Dorset, and monomethylethanolamine from Kodak Ltd., London. L-α-Glycerophosphate, as the sodium salt, was a gift from Dr R. W. Estabrook.